Aberrant Chromatin Remodeling by Retinoic Acid Receptor α Fusion Proteins Assessed at the Single-Cell Level[□]

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Acute promyelocytic leukemia (APL) is characterized by specific chromosomal translocations, which generate fusion proteins such as promyelocytic leukemia (PML)-retinoic acid receptor (RAR) α and promyelocytic leukemia zinc finger (PLZF)-RAR α (X-RAR α). In this study, we have applied *lac* operator array systems to study the effects of X-RAR α versus wild-type RAR α on large-scale chromatin structure. The targeting of these enhanced cyan fluorescent protein-*lac* repressor-tagged RAR α -containing proteins to the gene-amplification chromosomal region by *lac* operator repeats led to local chromatin condensation, recruitment of nuclear receptor corepressor, and histone deacetylase complex. The addition of retinoic acid (RA) induced large-scale chromatin decondensation in cells expressing RAR α ; however, cells expressing X-RAR α , especially PML-RAR α , demonstrated insensitive response to this effect of all-*trans* retinoic acid (ATRA). Although we did not reveal differences in RA-dependent colocalization of either silencing mediator for retinoid and thyroid or steroid receptor coactivator (SRC)-1 with RAR α versus X-RAR α , the hormone-independent association between SRC-1 and X-RAR α on the array has been identified. Rather, compared with cells expressing RAR α , fluorescence recovery after photobleaching of live transfected cells, demonstrated decreased mobility of SRC-1 on the X-RAR α -bound chromatin. Thus, the impaired ability of APL fusion proteins to activate gene transcription in response to ATRA corresponds to their reduced ability to remodel chromatin, which may link to their ability to impair the mobility of key nuclear receptor coregulators.

INTRODUCTION

The protein products that arise from chromosomal translocations are critical factors in carcinogenesis, especially in leukemia. Chromosomal translocations that occur in leukemic cells frequently involve genes for transcription factors, leading to the formation of chimeric proteins capable of interfering in cell signaling pathways that control important aspects of growth, differentiation, and survival within hematological cells (Look, 1997). Acute promyelocytic leukemia (APL) comprises 10% of acute myeloid leukemia, and it is most commonly associated with the specific reciprocal chromosomal translocation involving the retinoic acid receptor (RAR) α gene on chromosome 17 and the promyelocytic leukemia (PML) gene on chromosome 15 or the promyelocytic leukemia zinc finger (PLZF) gene on chromosome 11 (Melnick and Licht, 1999; Dong *et al.*, 2003a). These APL-specific chromosomal abnormalities generate PML-RAR α or PLZF-RAR α chimeric genes, which have been

demonstrated to play a key role in leukemogenesis by interfering with differentiation of bone marrow myeloid cells, causing arrest at the promyelocyte stage (Zelent *et al.*, 2001).

Numerous studies have been performed with the goal of understanding how chimeric RAR α -containing proteins contribute to leukemogenesis (Melnick and Licht, 1999; Zelent *et al.*, 2001; Lallemand-Breitenbach *et al.*, 2005). A critical contribution to this process made by the RAR α fusion partners is the ability to mediate homodimerization or oligomerization of the chimeric protein (Kwok *et al.*, 2006; Licht, 2006). However, the precise consequences of homodimerization in terms of leukemogenesis remain uncertain. One possibility supported by substantial *in vitro* data is that homodimerization results in binding to retinoic acid response element (RARE) sites required for normal myeloid differentiation, thereby competitively inhibiting binding to these sites by normal RAR α /retinoid X receptor (RXR) α heterodimers. An alternative explanation is that homodimerized chimeric RAR α -containing proteins interfere with expression of genes critical for normal myeloid differentiation by mislocalizing within the nucleus, resulting in spatiotemporal sequestration of nuclear coregulators essential for their expression (Dong *et al.*, 2004). Either spatiotemporal sequestration alone or competitive inhibition combined with the aberrant ligand-dependent interactions of X-RAR α with nuclear coactivators and corepressors may result in impaired chromatin remodeling and repression of transcription (Lin *et al.*, 2001).

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To explore the possibility in vivo that chimeric RAR α -containing fusion proteins contribute to leukemogenesis by impairing chromatin remodeling at the single-cell level, we used *lac* operator-*lac* repressor tethering systems, A03_1 and RRE_B1 cells, which contain multiple integrated copies of a high-affinity-binding site for the *lac* repressor, to directly visualize the effect of wild-type RAR α compared with PML-RAR α and PLZF-RAR α (X-RAR α) on large-scale chromatin dynamics. In A03_1 cell line system, multiple copies of the *lac* operator were engineered into the genome of Chinese hamster ovary (CHO) cells, and, together with the surrounding genomic sequences, they were amplified to produce a 90-Mb heterochromatic region or array (Robinett *et al.*, 1996; Li *et al.*, 1998). The expression of a chimeric protein containing the *lac* repressor and the activation domain of viral transcription factor VP16 in these A03_1 cells resulted in recruitment of the protein to the *lac* operator array and large-scale chromatin decondensation (Belmont *et al.*, 1999a; Tumber *et al.*, 1999; Belmont, 2001).

Interrogation of the effects of wild-type RAR α versus X-RAR α gene products on chromatin structure by using this *lac* repressor-tethering system revealed that expression of either cyan fluorescent protein (CFP)-LacR-tagged wild-type RAR α or X-RAR α protein results in 60% condensation of the array. However, compared with wild-type RAR α , cells expressing X-RAR α proteins, in particular PML-RAR α , were insensitive to normalization of the array volume when exposed to all-*trans* retinoic acid (ATRA). This aberrant response to ATRA did not correlate with abnormalities in the ability of X-RAR α to modulate their association with nuclear coactivators or corepressors on the array; rather, it correlated with the reduced mobility of coactivators on the array compared with expression of wild-type RAR α . Thus, the findings of this study support the hypothesis that expression of X-RAR α results in aberrant ATRA-mediated chromatin remodeling, possibly due to reduced mobility of critical nuclear coactivators.

MATERIALS AND METHODS

Reagents and Cell Lines

ATRA was obtained from Sigma-Aldrich (St. Louis, MO). A03_1 cell and RRE_B1 cells, which were kindly provided by Dr. Belmont (Robinett *et al.*, 1996; Li *et al.*, 1998), were maintained at 37°C with 5% CO₂ in customized F-12 Ham's media with 10% dialyzed fetal bovine serum (FBS; HyClone Labs) and methotrexate (0.3 μ M for A03_1 cells and 10 μ M for RRE_B1 cells), without phenol red, hypoxanthine and thymidine (Li *et al.*, 1998; Stenoien *et al.*, 2001). 293T cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum. HeLa cells were maintained in Opti-MEM 1 media (Invitrogen) with 4% FBS (Dong and Twardy, 2002; Dong *et al.*, 2004).

Vector Constructs

The CFP-LacR construct was made by inserting the LacR (*lac* repressor) sequence, amplified from polymerase chain reaction (PCR)-reaction, into the BglII-digested pECFP-C1 (Clontech, Mountain View, CA). The CFP-LacR-RAR α , CFP-LacR-PML-RAR α , and CFP-LacR-PLZF-RAR α constructs were made by inserting the LacR fragment into constructs CFP-RAR α , CFP-PML-RAR α , and CFP-PLZF-RAR α , respectively, described previously (Dong *et al.*, 2004). The silencing mediator for retinoid and thyroid (SMRT) and histone deacetylase 3 (HDAC3) cDNA fragments removed from a pCMX expression vector (Chen and Evans, 1995) and pcDNA3 expression vector (Invitrogen) (Yang *et al.*, 1996) were subcloned into pEYFP-C1 vector (Clontech) to make yellow fluorescent protein (YFP)-tagged SMRT and YFP-tagged HDAC3 expression vector, respectively. The YFP-tagged steroid receptor coactivator (SRC)-1 and RXR α expression vectors (in pEYFP-C1; Clontech) have been reported previously (Stenoien *et al.*, 2001; Dong *et al.*, 2004). The YFP-tagged RAR α , PML-RAR α , and PLZF-RAR α expression vectors were made from CFP-RAR α , CFP-PML-RAR α , and CFP-PLZF-RAR α (Dong *et al.*, 2004), respectively, by a swap involving placement of the cDNA for RAR α , PML-RAR α , and PLZF-RAR α into the pEYFP-C1 expression vector (Clontech). The YFP sequence within YFP-tagged SMRT vector was replaced with mCherry sequence from mCherry expression vector (in pRSET-B; Invitrogen) (Shaner *et al.*, 2005), to make cherry fluorescent protein (ChFP)-tagged SMRT expression vector. The fragment consisting of eight copies of *lac* operator (LacO) sequence, which was cut from vector p3216PECMS2 β (Prasanth *et al.*, 2005), was placed into pGL3-promoter vector (Promega, Madison, WI) with appropriate restriction enzymes, to make 8 $\times$ LacO luciferase reporter construct. All plasmid constructs were confirmed by DNA sequencing and immunoblotting (Figure 1A).

Cell Transfection, Immunoblotting, Luciferase Assay, and Gel-Shift DNA-Binding Assays

For transient transfections, HeLa cells, 293T cells, A03_1 cells, and RRE_B1 cells were grown in six-well plates to 60–70% confluence. The cells were transiently transfected with the indicated expression vectors, reporter genes, or a combination by using GeneJuice (Novagen, Madison, WI) as reported previously (Dong and Twardy, 2002). Twenty-four to 48 h after transfection, cells were lysed in lysis buffer for either immunoblotting or luciferase assay as described previously (Dong *et al.*, 1996; Dong and Twardy, 2002; Dong *et al.*, 2003b, 2004). Equivalent amounts of protein were electrophoresed on 7.5% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membrane developed using antibody against RAR α (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) or green fluorescent protein (GFP) (Dong *et al.*, 2003b).

For the gel-shift DNA-binding assays, CFP-tagged or CFP-LacR-tagged RAR α , PML-RAR α , and PLZF-RAR α proteins were expressed in 293T cells. Gel shift assays were performed as described previously (Dong *et al.*, 1996; Dong and Twardy, 2002) using 20 μ g of whole cell extracts of 293T cells transfected with the indicated constructs. Briefly, cell extracts were preincubated for 10 min at room temperature in the 20 μ l of reaction buffer, which contained 20 mM HEPES, pH 7.9, 0.5 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 50 mM KCl, and 1 μ g of poly(dI-dC). Radiolabeled *lac* operator or RARE (DR5G) duplex oligonucleotide was added and incubated for 50 min. The *lac* operator double-stranded oligonucleotide contains the sequence (top strand) 5'-TGTGGAATTGTGAGCGGATAACAATT-3' (Sasmor and Betz, 1990). The sequences of the RARE is as follows: 5'-GGGTAGGGTCACCGAAAGGTCACCTCG-3' (Dong *et al.*, 1996). One microgram of antibody against RAR α was included in the reaction for supershift experiments (Figure 1, C and D). Protein-DNA complexes were separated on 5% polyacrylamide gels equilibrated in 0.25 $\times$ Tris borate-EDTA. Gels were dried, exposed, and analyzed on the PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Fluorescence Microscopy and Fluorescence Recovery after Photobleaching (FRAP)

For fixed cell experiments, A03_1 and RRE_B1 cells were plated onto poly-D-lysine-coated coverslips in 24-wells plate and cultured in customized F-12 Ham's media with 10% charcoal-dextran treated FBS (Hyclone Laboratories). After 24 h, cells were transfected with the indicated CFP-, YFP-, or ChFP-labeled constructs using GeneJuice (Novagen) as described previously (Dong *et al.*, 2004). Forty-eight hours after transfection, cells were fixed in 4% formaldehyde in PEM buffer [80 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.8, 5 mM EGTA, and 2 mM MgCl₂] and quenched in 1 mg/ml NaBH₄ (Sigma-Aldrich) in PEM buffer. The A03_1 and/or RRE_B1 cells were stained with 1 μ g/ml 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 min before mounting in Slow Fade reagent (Invitrogen). Deconvolution microscopy was performed on a Zeiss Axiovert S100 TV microscope (Carl Zeiss, Thornwood, NY) by using DeltaVision restoration and microscopy system (Applied Precision, Issaquah, WA) as described previously (Stenoien *et al.*, 2000). A series of Z-sections were imaged and deconvolved with the DeltaVision constrained iterative algorithm. For quantitative measurement, the DeltaVision software was used to measure the volume of the three-dimensional images of the LacO arrays of A03_1 cells, which were built by the software using the series of Z-sections of each array. The *lac* operator arrays within RRE_B1 cells were much more extended, and instead of measuring the volume of the array, the array occupied chromatin area was measured using the Z-section containing the largest array area. Both the volume and area measurements were exported, analyzed, and graphed in Excel (Microsoft, Redmond, WA).

For dual-FRAP analysis, cells were plated onto Delta T dishes (Bioprotech, Butler, PA) 24 h before transfection at a concentration of 2×10^5 cells per dish. The expression of CFP- or YFP-tagged plasmids was performed using GeneJuice (Novagen) as described previously (Dong *et al.*, 2004; Qiu *et al.*, 2006). FRAP was performed on the heated stage of an LSM 510 confocal laser scanning microscope (Carl Zeiss) with a 63 $\times$, 1.4 numerical aperture Plan-Apochromat oil immersion objective. For both CFP and YFP fluorescent signals, the bleach was accomplished with the laser set at 458 and 514 nm and at 70% laser output for 30 iterations of a small circle of each *lac* operator array in A03_1 cells. Simultaneous images corresponding to the CFP and YFP fluorescence were obtained before and at different time intervals after the bleach by using the multitracking function of the microscope (typically 5 prebleach and 100 postbleach frames). Fluorescent intensities of regions of interest (ROI) were measured with LSM software, and exported to Excel for analysis. Fluorescent intensities of the ROI were corrected for background and normalized to the mean of the last three prebleach values by using the following equation

Figure 1. Characterization of CFP-LacR-containing constructs. (A) Western blot analysis of CFP-LacR-tagged RAR α and X-RAR α (PML-RAR α and PLZF-RAR α) proteins expressed in 293T cells by using antibody against RAR α . (B–D) Gel-shift assay analysis of whole cell extracts of 293T cells transiently transfected with CFP-LacR-RAR α or CFP-LacR-X-RAR α (PML-RAR α and PLZF-RAR α) alone or plus RXR α , as indicated, were performed using *lac* operator (B) or RARE sequence (C and D). The filled arrowheads indicate the positions of the CFP-LacR-X-RAR α homodimer/RARE complex. The empty arrowheads indicate the positions of the CFP-LacR-RAR α /RXR α heterodimer/RARE complex or CFP-LacR-X-RAR α /RXR α /RARE complex. Extracts of 293T cells transiently transfected with CFP-PML-RAR α and CFP-PLZF-RAR α were included as size controls (C). The RAR α antibody was used for supershift experiments as indicated (B and D). (E) Luciferase activity in lysates of HeLa cells transiently transfected with CFP-LacR-tagged RAR α or X-RAR α proteins incubated in the absence or presence of ATRA at 10⁻⁶ M. The amounts of plasmid DNA used per well were 250 ng of 8 $\times$ LaCo luciferase reporter vector, 500 ng of CFP-LacR-tagged expression vector and 200 ng of β -galactosidase expression vector as transfection control. Luciferase activity within cell lysates was measured in a luminometer, expressed in arbitrary units and normalized to the transfection control. Data presented are the mean $\pm$ SD of at least three independent experiments. (F) Fluorescence microscopy of representative cells demonstrating nuclear localization and distribution of CFP-tagged or CFP-LacR-tagged RAR α and X-RAR α protein within transfected HeLa cells or A03_1 cells transiently transfected with the indicated vector construct.

for each time point: $(ROI_{bleach,t} - ROI_{background}) / (ROI_{bleach, prebleach} - ROI_{background})$. The normalized recovery curve was adjusted so that the lowest point in the curve was assigned a value of 0, and the highest point

was assigned a value of 1. The $t_{1/2}$ of observed recovery was defined as the time required for reaching half-maximum recovery, and it was calculated from the normalized recovery curve.

Statistical Analysis

LacO array volume and area data were transformed by taking logarithms to stabilize variances and reduce skewness. Summary statistics (geometric means and 95% confidence intervals) on the raw scale were computed by back-transforming the means and 95% confidence intervals of the transformed data. One-way analysis of variance (ANOVA) was used to determine whether there were any differences between groups, followed by specific linear contrasts to identify which groups differed. Analyses were conducted with SAS, version 9.1 (SAS Institute, Cary, NC).

RESULTS

Generation of Functional CFP-LacR-tagged Wild-Type RAR α and X-RAR α

To study the intracellular function of wild-type RAR α , PML-RAR α , and PLZF-RAR α in the context of chromatin at the single-cell level, we created vectors expressing CFP-LacR (*lac* repressor) chimeras fused to the N-terminal ends of RAR α , PML-RAR α , and PLZF-RAR α . Immunoblot analysis of whole cell extracts from transfected 293T cells showed that all CFP-LacR-tagged constructs encoded proteins of expected size (Figure 1A). To determine whether these CFP-LacR-tagged proteins could bind to *lac* operator DNA sequence contained within the *lac* operator array in A03_1 and RRE_B1 cell lines (Li *et al.*, 1998), we performed gel-shift assays by using radiolabeled *lac* operator duplex oligonucleotide. As expected, all of the CFP-LacR-tagged proteins tested bound to *lac* operator duplexes and could be supershifted by antibody against RAR α (Figure 1B). To be certain that the addition of CFP-LacR to RAR α , PML-RAR α , or PLZF-RAR α did not significantly alter the functional characteristics of the RAR α -containing portion of the construct, we examined the DNA binding activity of each using gel-shift assays with RARE (DR5G); each bound to RARE not only as a homo-oligomer, but also as a hetero-oligomer with RXR α , similar to results reported by us and other investigators with each of the untagged counterparts (Figure 1, C and D) (Dong and Tweardy, 2002; Zeisig *et al.*, 2007). In addition, these RAR α -related hetero-oligomer and homo-oligomer complexes were further studied using the LacO array, A03_1 cell system. A03_1 cells were cotransfected with CFP-LacR-tagged RAR α , PML-RAR α , and PLZF-RAR α plus their YFP-tagged counterpart or YFP-tagged RXR α (Supplemental Figure 1). As expected, YFP-tagged RXR α bound to all of CFP-LacR-tagged RAR α constructs bound to the array (Supplemental Figure 1A). Also, YFP-tagged X-RAR α (PML-RAR α and PLZF-RAR α) were able to form homo-oligomer complexes with their respective CFP-LacR-tagged counterpart bound to the array (Supplemental Figure 1, C and D) in contrast to YFP-tagged wild-type RAR α , which did not bind to CFP-LacR-RAR α bound to arrays (Figure 1B). These results confirm at the single-cell level previous *in vitro* studies by us and others (Dong *et al.*, 1996; Dong and Tweardy, 2002; Kwok *et al.*, 2006; Zeisig *et al.*, 2007).

To assess whether CFP-LacR-tagged proteins could bind the *lac* operon and activate transcription in an RAR α ligand-dependent manner, we examined the transcriptional activity of CFP-LacR-RAR α and CFP-LacR-X-RAR α by using a luciferase reporter system described previously (Dong *et al.*, 1996; Dong and Tweardy, 2002) modified to contain multiple copies of the *lac* operator. HeLa cells were cotransfected with expression plasmid containing CFP-LacR-RAR α (Figure 1E, lanes 1 and 2), CFP-LacR-PML-RAR α (Figure 1E, lanes 3 and 4), CFP-LacR-PLZF-RAR α (Figure 1E, lanes 5 and 6), or control CFP-LacR (Figure 1E, lanes 7 and 8) plus the 8 $\times$ *lac* operator-containing luciferase reporter vector in the absence or presence of ATRA, a ligand for RAR α . The transcriptional activities of the CFP-LacR-RAR α and CFP-LacR-X-RAR α

were shown to be retinoic acid inducible—ninefold by wild-type RAR α , fivefold by PML-RAR α , and 12-fold by PLZF-RAR α . Of note, in the absence of ATRA, CFP-LacR-RAR α could activate this reporter twofold (Figure 1E, lane 1 vs. lane 7), whereas PML-RAR α and PLZF-RAR α repressed basal activation of this reporter by two- and threefold, respectively. These results are in agreement with those published by us and other investigators using untagged RAR α and X-RAR α and a luciferase reporter construct driven by the RAR β promoter (Dong *et al.*, 1996, 2003b; Dong and Tweardy, 2002). Finally, we examined whether the addition of LacR altered the cellular localization of RAR α or X-RAR α . After transient expression in HeLa cells, each LacR-tagged protein localized predominantly within the nucleus in a pattern identical to that reported previously by us for their CFP-tagged counterparts (Figure 1F) (Dong *et al.*, 2004). Thus, addition of the LacR tag conferred the ability of the LacR-tagged proteins could bind to a *lac* operator containing reporter construct and to activate transcription in a ligand-dependent manner, but it did not interfere with the RARE binding ability of CFP-RAR α and CFP-X-RAR α nor with their cellular localization and distribution.

Effect of CFP-LacR-RAR α and CFP-LacR-X-RAR α Binding on the *lac* Operator Array

To assess the effect of wild-type RAR α versus PML-RAR α or PLZF-RAR α on large-scale mammalian chromatin structure at the single-cell level, we made use of a CHO cell line, A03_1, in which multiple copies of the *lac* operator were engineered to produce an integrated 90-Mb heterochromatic array within the genome (Li *et al.*, 1998). The molecular organization of this array consists of 400-kb repeats of the 14-kb vector insert that contains the *lac* operator repeat and the dihydrofolate reductase selectable marker (Robinett *et al.*, 1996; Li *et al.*, 1998). This *lac* operator array system has been applied by Dr. Belmont's group for studying the transcriptional responses of chromatin structure during transcriptional activation with enhanced green fluorescent protein (EGFP)-*lac* repressor (LacR)-VP16 (Tumbar *et al.*, 1999), which is a potent viral transcription activator protein, and EGFP-*lac* repressor-estrogen receptor (ER) (Nye *et al.*, 2002). Targeting this VP16 fusion protein to the *lac* operator array in A03_1 cells resulted in visualization of chromatin decondensation within 15 min, accompanied by the recruitment of histone acetyltransferases and by histone hyperacetylation (Tumbar *et al.*, 1999). In our studies, CFP-LacR transiently transfected into A03_1 cells distributed almost exclusively on the array consistent with high-affinity binding of LacR to the LacO and the previous results of others (Li *et al.*, 1998; Stenoien *et al.*, 2001). The array volume was 17.25 μm^3 (95% confidence interval [CI] = 12.26–24.31) in cells untreated with ATRA, and it did not change in cells treated with ATRA at 10^{-7} or 10^{-6} M (Figure 2). Similar to CFP-LacR, CFP-LacR-RAR α , and CFP-LacR-X-RAR α (CFP-LacR-PML-RAR α , and CFP-LacR-PLZF-RAR α) proteins transiently expressed in A03_1 cells each were distributed almost exclusively on the array. However, in contrast to CFP-LacR, the array volumes in cells transfected with CFP-LacR-RAR α (7.19 μm^3 ; 95% CI = 5.91–8.76), CFP-LacR-PML-RAR α (7.38 μm^3 ; 95% CI = 6.35–8.58), and CFP-LacR-PLZF-RAR α (7.42 μm^3 ; CI = 6.22–8.85) were reduced in each instance by ~60% within cells untreated with ligand ($p < 0.001$ for each). To confirm these results in a cell line containing a *lac* operator array that is more euchromatin-like, we repeated the experiment using the RRE_B1 cell line. The RRE_B1 cell line, like A03_1, is derived from CHO cells, and it contains amplified genomic domains consisting of *lac* operator bind-

Figure 2. Effect of CFP-LacR-RAR α or CFP-LacR-X-RAR α binding on the *lac* operator array volume within A03_1 cells. (A) A03_1 cells were transfected with CFP-LacR-tagged RAR α and X-RAR α , followed with or without ATRA treatment. The volume of the arrays was determined as described under *Materials and Methods* and presented as the geometric mean $\pm$ 95% CI. (B) Representative images are shown for arrays bound by CFP-LacR (first row panels), CFP-LacR-RAR α (second row panels), CFP-LacR-PML-RAR α (third row panels), and CFP-LacR-PLZF-RAR α (bottom row panels) within A03_1 cells incubated without ATRA (left column panels), with ATRA at 10^{-7} M for 3 h (middle column panels) and with ATRA at 10^{-6} M for 3 h (right column panels). The number of cells examined, the geometric mean (GM), and a histogram showing the distribution of array volumes is presented below each representative image. The nuclei were stained with DAPI (blue).

Figure 3. Effect of CFP-LacR-RAR α and CFP-LacR-X-RAR α expression and ATRA on the localization and distribution of nuclear receptor corepressor SMRT, HDAC3, and nuclear receptor coactivator SRC-1 within A03_1 cells. Cells cotransfected with YFP-SMRT (A), YFP-HDAC3 (B), or YFP-SRC-1 (C), and CFP-LacR-RAR α or CFP-LacR-X-RAR α was subjected to deconvolution microscopic analysis after treatment with ATRA for 3 h at the indicated concentrations. Nuclei were visualized by DNA staining with DAPI (blue). DAPI and CFP, YFP, or merged CFP and YFP images from representative nuclei are shown in the top left, top right, and bottom left panels. The percentage of cells (*y*-axis) that showed complete binding (yellow bar), partial binding with partial diffusion (purple bar), and diffusion (blue bar) are shown in the bottom right panel. The number at the top of bar graphs represent the number of nuclei examined for each group; the data presented are the combined results of three independent experiments.

ing sites, in an extended, often fibrillar conformation (Robinet *et al.*, 1996; Verschure *et al.*, 2005). Previous studies that targeted an EGFP-LacR-HP1 construct onto this array caused local chromatin condensation (Verschure *et al.*, 2005). Identical to our results with A03_1 cells, we observed array condensation in RRE_B1 cells transfected with each CFP-LacR-RAR α -containing construct compared with CFP-LacR (Supplemental Figure 2). Thus, results in both array-containing cell lines are consistent with the hypothesis that wild-type RAR α , PML-RAR α , and PLZF-RAR α each recruit corepressors with histone deacetylase activity (Figure 3B).

Addition of 10^{-7} M ATRA to cells containing CFP-LacR-RAR α increased the array volume by twofold consistent

with release of corepressors and/or recruitment of coactivators with histone acetylase activity (Figure 2). In contrast, addition of 10^{-7} M ATRA to cells expressing CFP-LacR-PML-RAR α or CFP-LacR-PLZF-RAR α did not increase the array volume (Figure 2). Increasing the ATRA concentration to 10^{-6} M did not increase the array volume in cells containing CFP-LacR-PML-RAR α , but it did increase the array volume in cells containing CFP-LacR-PLZF-RAR α by 64% to a value indistinguishable from the array volume in cells expressing CFP-LacR-RAR α and incubated in 10^{-6} M ATRA (Figure 2). Very similar results were obtained by using euchromatin-like RRE_B1 cell line (Supplemental Figure 2). The finding that the arrays in cells expressing CFP-LacR-

Figure 3. Continued.

PML-RAR α were more resistant to the ATRA-induced increase in volume than arrays in cells expressing CFP-LacR-PLZF-RAR α was somewhat unexpected based on results of clinical studies demonstrating that APL patients with PML-RAR α -positive disease are ATRA responsive, whereas patients with PLZF-RAR α -positive disease are ATRA resistant (Melnick and Licht, 1999; Zelent *et al.*, 2001).

We and others have reported that the protein products of X-RAR α fusion genes were degraded within cells upon retinoic acid treatment (Koken *et al.*, 1999; Melnick and Licht, 1999; Dong and Tweardy, 2002). To assess whether differences in protein stability participated in the variable ATRA-induced increase in array volumes, we performed immunoblotting analysis of whole cell extracts from cells transiently transfected with CFP-LacR-tagged RAR α and X-RAR α before and after ATRA treatment (Supplemental Figure 3). The level of each of the RAR α -containing proteins in A03_1 cells incubated with ATRA was virtually unchanged, indicating that ATRA-mediated changes in protein stability did not contribute to changes in array volumes.

Effect of CFP-LacR-RAR α and CFP-LacR-X-RAR α Expression and ATRA on the Distribution of Nuclear Receptor Corepressor SMRT and Coactivator SRC-1 within A03_1 Cells

The *lac* operator array A03_1 cell system has been used previously to study nuclear receptor biology (Stenoien *et al.*, 2001), and it revealed interactions between the ER and both SRC-1 and CREB-binding protein (CBP) on the array within A03_1 cells. Transient triple-transfection of A03_1 cells with CFP-LacR-tagged RAR α or X-RAR α plus YFP-SRC-1 and ChFP-SMRT construct established that each nuclear receptor coregulator demonstrated ATRA-dependent interactions with each CFP-LacR-tagged RAR α -containing construct on the *lac* operator array in the expected manner, confirming

earlier studies at the single-cell level (Supplemental Figure 4).

To determine whether the tagged RAR α -containing constructs recruited nuclear corepressors to the array and whether this contributed to ATRA-induced changes in the *lac* operator array volume, we imaged A03_1 cells coexpressing YFP-SMRT and each of the CFP-LacR-containing constructs. Coexpression of YFP-SMRT and CFP-LacR in cells untreated with ATRA revealed CFP-LacR localized to the array, whereas YFP-SMRT distributed diffusely throughout the nucleus, and it did not colocalize with CFP-LacR on the array (data not shown). In contrast, coexpression of YFP-SMRT and CFP-LacR-RAR α in A03_1 cells untreated with ATRA revealed virtually all the YFP-SMRT colocalized with CFP-LacR-RAR α on the array (Figure 3A) consistent with findings summarized above of smaller array volumes in cells expressing CFP-LacR-tagged RAR α versus CFP-LacR alone. Treatment of these cotransfected cells with ATRA at either 10⁻⁷ or 10⁻⁶ M resulted in partial or complete dissociation of YFP-SMRT from the array, which correlated with the increase in array volume observed in cells transfected with CFP-LacR-RAR α and treated with ATRA at these concentrations (Figure 3A). Coexpression of YFP-SMRT with either CFP-LacR-PML-RAR α or CFP-LacR-PLZF-RAR α in A03_1 cells untreated with ATRA resulted in virtually all the YFP-SMRT colocalized with CFP-LacR-X-RAR α on the array (Figure 3A). Unexpectedly, treatment of both sets of cotransfected cells with ATRA at either 10⁻⁷ or 10⁻⁶ M revealed partial or complete dissociation of YFP-SMRT from the array, similar to the results obtained with ATRA-treated cells cotransfected with YFP-SMRT and CFP-LacR-RAR α . Thus, YFP-SMRT associated with RAR α -containing constructs on the arrays and this association correlated to array volumes in the absence of ATRA. Furthermore, ATRA-induced dissociation of YFP-SMRT from the array correlated well with the ATRA-induced increase in array volume in cells expressing CFP-

Figure 4. Nuclear mobility of YFP-SRC-1 and YFP-SMRT in A03_1 cells expressing CFP-LacR-RAR α or CFP-LacR-X-RAR α . Dual-FRAP analysis was performed in A03_1 cells cotransfected with YFP-SMRT and CFP-LacR-RAR α (top), YFP-SMRT and CFP-LacR-PML-RAR α (middle), and YFP-SMRT and CFP-LacR-PLZF-RAR α (bottom) (A) after incubation with ATRA at 10^{-7} M for 30 min and in A03_1 cells cotransfected with YFP-SRC-1 and CFP-LacR-RAR α (top), YFP-SRC-1 and CFP-LacR-PML-RAR α (middle), and YFP-SRC-1 and CFP-LacR-PLZF-RAR α (bottom) (B) after incubation with ATRA at 10^{-7} M for 30 min. Images show single Z-sections, and they were obtained before bleaching and at the indicated time points after bleaching.

LacR-tagged wild-type RAR α ; however, this was not the case for cells expressing either CFP-LacR-PML-RAR α or CFP-LacR-PLZF-RAR α .

HDAC3 is known to form SMRT-HDAC3 functional complexes (Li *et al.*, 2002). Examination of A03_1 cells cotransfected with the CFP-LacR construct revealed HDAC3 present at very low levels in the amplified chromosomal region (data not shown). A03_1 cells cotransfected with YFP-HDAC3 and either CFP-LacR-RAR α or CFP-LacR-X-RAR α demonstrated virtually all of the YFP-HDAC3 partially colocalized to the *lac* operator array in the absence of ATRA (Figure 3B). After addition of ATRA, YFP-HDAC3 completely dissociated from the array. Thus, YFP-HDAC3 followed the identical pattern of array colocalization as YFP-SMRT.

To determine whether the tagged RAR α -containing constructs recruited nuclear coactivators to the array and whether this contributed to changes in the *lac* operator array volume, we imaged A03_1 cells coexpressing YFP-SRC1 and CFP-LacR, CFP-LacR-RAR α , CFP-LacR-PML-RAR α or CFP-LacR-PLZF-RAR α . Coexpression of YFP-SRC-1 and CFP-LacR in A03_1 cells revealed CFP-LacR localized to the array, whereas YFP-SRC-1 distributed diffusely throughout the nucleus; no colocalization of YFP-SRC-1 with CFP-LacR on the array was observed (data not shown). Coexpression

of YFP-SRC-1 and CFP-LacR-RAR α in A03_1 cells untreated with ATRA revealed CFP-LacR-RAR α localized to the array, and most of the YFP-SRC-1 distributed diffusely throughout the nucleus (Figure 3C). Examination of cotransfected cells treated with ATRA at either 10^{-7} or 10^{-6} M revealed nearly complete localization of YFP-SRC-1 on the array, which correlated well with the increase in array volume observed in cells transfected with CFP-LacR-RAR α summarized above (Figure 3C). Coexpression of YFP-SRC-1 with either CFP-LacR-PML-RAR α or CFP-LacR-PLZF-RAR α in A03_1 cells untreated with ATRA resulted in YFP-SRC-1 distributed diffusely throughout the nucleus. However, the increasing complete binding of coactivator SRC-1 into X-RAR α -bound chromatin area (23% PML-RAR α - and 44% PLZF-RAR α -expressing cells, in contrast to only 7% wild-type RAR α -expressing cells) was observed, indicating a ligand-independent association between SRC-1 and X-RAR α (Figure 3C). Treatment of both sets of cotransfected cells with ATRA at either 10^{-7} or 10^{-6} M revealed nearly complete localization of YFP-SRC-1 on the array, again similar to the results with cells cotransfected with YFP-SRC-1 and CFP-LacR-RAR α . Thus, ATRA treatment of cotransfected A03_1 cells induced association of SRC-1 with all tagged RAR α constructs on the array. Furthermore, although ATRA-induced colocalization of YFP-SRC-1 with CFP-LacR-tagged constructs on the ar-

Table 1. Fluorescence recovery $t_{1/2}$ of YFP-SRC-1 and YFP-SMRT proteins

No.	Construct	$t_{1/2}$ (s) + ATRA (10^{-7} M) for 30 min
1	COLYFP-SMRT with CFP-LacR-RAR α	13.57 ± 4.53 s, n = 23
2	YFP-SMRT with CFP-LacR-PML-RAR α	13.50 ± 4.21 s, n = 30
3	YFP-SMRT with CFP-LacR-PLZF-RAR α	22.76 ± 7.86 s, n = 27
4	YFP-SMRT alone	0.69 ± 0.14 s, n = 12
5	YFP-SRC-1 with CFP-LacR-RAR α	17.80 ± 4.30 s, n = 20
6	YFP-SRC-1 with CFP-LacR-PML-RAR α	26.07 ± 8.35 s, n = 21
7	YFP-SRC-1 with CFP-LacR-PLZF-RAR α	25.80 ± 9.89 s, n = 24
8	YFP-SRC-1 alone	1.11 ± 0.22 s, n = 12

p < 0.001 for 1, 2 versus 3; p = 0.48 for 1 versus 2; p < 0.001 for 5 versus 6, 7; and p = 0.46 for 6 versus 7.

rays correlated with an increase in array volume in cells expressing tagged wild-type RAR α , this was not the case for either tagged PML-RAR α or PLZF-RAR α . Thus, when assessed at the single-cell level, neither association nor dissociation of either SMRT/HDAC3 or SRC-1 was sufficient to explain differences in ATRA-induced array volumes between wild-type RAR α and X-RAR α .

Nuclear Mobility of YFP-SRC-1 and YFP-SMRT in A03_1 Cells Expressing CFP-LacR-RAR α or CFP-LacR-X-RAR α

We previously demonstrated that X-RAR α protein expression resulted in aberrant localization and reduced mobility of key nuclear receptor coregulators (Dong *et al.*, 2004). To assess whether reduced mobility of nuclear receptor corepressors contributed to aberrant ATRA-mediated chromatin remodeling in cells expressing X-RAR α , we performed dual-FRAP of A03_1 cells cotransfected with YFP-SMRT and CFP-LacR-tagged wild-type RAR α or X-RAR α . In the absence of ATRA, the mobility for YFP-SMRT is very slow ($t_{1/2}$ > 10 min; data not shown). However, as shown in Figure 3A, treatment with ATRA at 10^{-7} M for 3 h resulted in nearly complete dissociation of YFP-SMRT from CFP-LacR-tagged RAR α and X-RAR α on the array. To obtain measurable recovery times, cells were examined after treatment with ATRA at this concentration for 30 min; nuclei selected for analysis were those in which the YFP signal was both on and off the array. After the 2-s bleach, there was little or no recovery of the CFP signal over 99 s (Figure 4A) or even over 20 min (data not shown), indicating that the LacR-containing proteins were essentially immobilized on the array due to the high-affinity binding of the *lac* repressor domain to the *lac* operator sites within the array. The mobility of YFP-SMRT localized on the CFP-LacR-RAR α -bound array assessed by its recovery $t_{1/2}$ was 13.57 ± 4.53 s, whereas the mobility of nonarray bound YFP-SMRT was very fast with recovery $t_{1/2} = 0.69 \pm 0.14$ s (Table 1). The mobility of YFP-SMRT localized on the CFP-LacR-PML-RAR α -bound array was essentially identical to that for CFP-LacR-RAR α ($t_{1/2} = 13.50 \pm 4.21$ s). In contrast, the mobility of YFP-SMRT on the CFP-LacR-PLZF-RAR α -bound array was reduced by 68% ($t_{1/2} = 22.76 \pm 7.87$ s; p < 0.001; Figure 4A and Table 1) compared with YFP-SMRT on the CFP-LacR-RAR α -bound array, indicating that YFP-SMRT bound more tightly to PLZF-RAR α than to wild-type RAR α . We demonstrated reduced intranuclear mobility of SMRT bound to PLZF-RAR α , which most likely reflects increased binding affinity of SMRT to PLZF-RAR α compared with

either wild-type RAR α or PML-RAR α . The biochemical and molecular basis for a higher affinity interaction between SMRT and PLZF-RAR α in vitro has been attributed to the PLZF POZ domain (Dong *et al.*, 1996; Lin *et al.*, 1998). Although consistent with these in vitro findings, our in vivo results do not explain the resistance to the ATRA-induced increase in array volumes in cells expressing both X-RAR α , in particular PML-RAR α (Figure 2).

To assess whether reduced mobility of nuclear receptor coactivators contributed to aberrant ATRA-mediated chromatin remodeling in cells expressing X-RAR α , we performed dual FRAP over the array in A03_1 cells coexpressing YFP-tagged coactivator SRC-1 and CFP-LacR-tagged wild-type RAR α or X-RAR α . Cells with YFP signal mostly on the array after treatment with ATRA at 10^{-7} M for 30 min were chosen for analysis. After the 2-s bleach, the CFP signal had no or little recovery as expected (Figure 4B). The mobility of YFP-SRC-1 not bound to the array is fast (recovery $t_{1/2} = 1.11 \pm 0.22$ s). However, the mobility of YFP-SRC-1 localized on the CFP-LacR-RAR α -bound array was slower ($t_{1/2} = 17.80 \pm 4.31$ s; Table 1) and even slower in cells containing CFP-LacR-PML-RAR α -bound arrays ($t_{1/2} = 26.07 \pm 8.35$ s; Table 1; p < 0.001) and in cells containing CFP-LacR-PLZF-RAR α -bound arrays ($t_{1/2} = 25.80 \pm 9.89$ s; Table 1; p < 0.001). These results indicate that YFP-SRC-1 bound more tightly to X-RAR α than to wild-type RAR α . Thus, reduced mobility of YFP-SRC-1 on X-RAR α -bound arrays correlated with resistance to the ATRA-induced increase in array volumes.

DISCUSSION

We used two integrated *lac* operator array cell systems (Belmont *et al.*, 1999b) to examine the hypothesis at the single-cell level that RAR α -containing APL fusion proteins remodel chromatin aberrantly compared with wild-type RAR α . Fluorescent microscopic examination of arrays after expression of CFP-LacR-tagged, RAR α -containing proteins demonstrated that each of the tagged proteins bound to arrays resulting in condensation of the array volume to less than half that of arrays bound by *lac* repressor alone. All-trans retinoic acid treatment resulted in array decondensation in cells expressing tagged wild-type RAR α . However, cells expressing tagged X-RAR α were insensitive to the array-decondensing effect of ATRA at different level (Figure 2).

Eukaryotic cells use multiple steps at several structural levels to effectively package their genome within the nucleus. It is now well established that unpacking or remodeling of chromatin structure locally is a critical step in the initiation of multiple chromosome functions notably transcription (Belmont *et al.*, 1999a). Whereas intense research in the past decade has provided a wealth of information regarding the biochemical basis for ligand-dependent transcriptional properties of RAR α (Chambon, 1995), much less is known about reorganization of local chromatin structure by RAR α protein and how this compares with X-RAR α oncogenic proteins. Our studies are the first to report the effects of RAR α -containing fusion proteins on chromatin structure and nuclear receptor coregulator recruitment in vivo at the single-cell level. The finding that expression of LacR-tagged RAR α within *lac* repressor-tethering cell systems in the absence of ligand results in *lac* operator array condensation is consistent with in vitro evidence that, in the absence of ligand, RAR α can recruit corepressor with histone deacetylase activity (Melnick and Licht, 1999). Cotransfection of *lac* operator array-containing cells with CFP-LacR-RAR α and YFP-SMRT or YFP-HDAC3 confirmed this at the

single-cell level. We also demonstrated that *lac* operator arrays bound by wild-type RAR α decondensed after incubation of cells in ATRA, consistent with *in vitro* evidence that ligand addition results in release of corepressors and recruitment of coactivators with histone acetylase activity (Licht, 2001, 2006; Zelent *et al.*, 2001; Melnick *et al.*, 2002). Examination of *lac* operator array-containing cells cotransfected with CFP-LacR-RAR α and YFP-SMRT or YFP-SRC-1 as well as cells transfected with all three constructs revealed that the addition of ATRA resulted in both the release of SMRT from the RAR α -bound *lac* operator array and the recruitment of coactivator SRC-1 at the single-cell level.

These studies provide intriguing insight at the single-cell level regarding how APL fusion proteins may function as oncogenes. Similar to wild-type RAR α , expression of both PML-RAR α and PLZF-RAR α proteins in *lac* repressor-tethering systems resulted in *lac* operator array condensation. In the absence of ligand, each also was shown to recruit nuclear receptor corepressor SMRT to the array, thereby confirming *in vivo* at the single-cell level previous *in vitro* studies that demonstrated the ability of corepressor to bind to APL fusion proteins in the absence of ligands (Lin *et al.*, 1998; Dong and Twardy, 2002). However, somewhat unexpectedly, based on previously reported responses in APL patients to ATRA (Melnick and Licht, 1999), cells containing PML-RAR α were more, not less, insensitive to ATRA-induced chromatin decondensation compared with cells containing PLZF-RAR α . In addition, the complete dissociation of corepressor SMRT from PLZF-RAR α -bound chromatin area in retinoic acid (RA)-treated cells was observed, which was in line with the RA-dependent transactivation effect by this tagged PLZF-RAR α , although we found the decreased SMRT nuclear mobility with chromatin-bound PLZF-RAR α in comparison with that with wild-type RAR α or PML-RAR α . The integration of our data with earlier findings, in which RA treatment led to PLZF-RAR α degradation (Koken *et al.*, 1999; Dong and Twardy, 2002), strongly suggests that PLZF-RAR α chimeric protein responds well to the treatment of retinoic acid. However, these effects were not accompanied by terminal differentiation or apoptosis of APL cells (Koken *et al.*, 1999). These findings suggest that the molecular basis for differences in clinical response to ATRA for these two forms of APL may lie beyond differences in ATRA-mediated changes in large-scale chromatin remodeling. Combined with the transgenic studies (He *et al.*, 2000), in which, Pandolfi's group found that double transgenic mice with RAR α -PLZF and PLZF-RAR α but PLZF-RAR α alone developed leukemia with typical APL features, our single-cell studies highlighted the importance of reciprocal RAR α -PLZF fusion protein, whose mRNA was existed in all t(11;17) APL patients (Melnick and Licht, 1999), in leukemogenesis as well as clinical RA resistance.

There is growing evidence that the dynamic exchange of proteins on chromatin is essential for transcriptional activators to gain access to chromatin and that controlling the exchange rate of a protein on chromatin might contribute to regulation of gene expression (Misteli, 2001). Although we found evidence of ATRA dependence of SMRT and SRC-1 interactions with APL fusion proteins *in vivo* using *lac* repressor-tethering system as reported previously *in vitro* (Lin *et al.*, 1998; Dong and Twardy, 2002), no differences were observed in the ATRA concentration dependence of the interactions of either SMRT or SRC-1 with X-RAR α compared with wild-type RAR α that would explain differences in ATRA-induced array decondensation. However, we found that chromatin-bound X-RAR α (PML-RAR α and PLZF-RAR α) fusion proteins associated with nuclear recep-

tor coactivator SRC-1 in a retinoic acid-independent way. Furthermore, the impaired ATRA-dependent decondensation in cells expressing X-RAR α correlated with reduced intranuclear mobility of SRC-1. Reduced intranuclear mobility presumably is due to increased binding affinity of SRC-1 to PML-RAR α and PLZF-RAR α . In line with our studies, very recently, Dr. Kao's group demonstrated that PML-RAR α oncogenic protein aberrantly interacts with nuclear receptor coactivator SRC-3 and CBP in a hormone-independent manner, which correlate with the ability of PML-RAR α to decrease the transcriptional activation by the glucocorticoid receptor and Notch signaling pathway (Reineke *et al.*, 2007). By binding nuclear receptor coactivators such as SRC-1, SRC-3, and CBP with higher affinity than wild-type RAR α , APL fusion proteins may contribute to transcriptional alterations resulting in diseases by reducing the availability of these coactivators to bind to sites on chromatin important for maintaining the normal transcriptome of the cell.

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